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The Nature of the Voltage-Dependent Conductance Induced by Alamethicin in Black Lipid Membranes

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Summary. Alamethicin induces a conductance in black lipid films which increases exponentially with voltage. At low conductance the increase occurs in discrete steps which form a pattern of five levels, the second and third being most likely. The conductance of each level is directly proportional to salt concentration, inversely proportional to solution viscosity, and nearly independent of voltage.

The probability distribution of the five steps is not a function of voltage, but as the voltage is increased, more levels begin to appear. These can be explained as superpositions of the original five, both in position and relative probability.

This suggests that the five levels are associated with a physical entity which we call a pore. This point of view is confirmed by the following measurements. The kinetic response of the current to a voltage step is first order, and shows an exponential increase in rate of pore formation and an exponential decrease in rate of pore disappearance with voltage. If these rates are statistical, the number of pores should fluctuate about a voltage-dependent mean. High conductance current fluctuations are too large to be explained by fluctuation in the number of pores alone. But if fluctuations among the five levels are included, the magnitude of the fluctuations at high conductance is accurately predicted.

Alamethicin adsorbs reversibly to the membrane surface, and the conductance at a fixed voltage depends on the ninth power of alamethicin concentration and on the fourth power of salt concentration, in the aqueous phase. In our bacterial phosphatidyl ethanolamine membranes, alamethicin added to one side of the membrane produces elevated conductance only when the voltage on that side is increased.

The functioning of excitable membranes, notably those of nerve and muscle cells, has been thought for some time to depend on voltage-dependent permeability changes for particular ions (Hodgkin & Huxley, 1952). In the case of nerve, the propagation of the action potential results from a transient

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increase in sodium permeability followed by an increase in the potassium permeability. Both increases are functions of voltage. In neither case is there any direct knowledge of the molecular mechanism by which the voltage controls the permeability.

There is, however, indirect evidence (for a review *see* Hille, 1970) that the permeability increase results from the opening of numerous small pores of two kinds in the membrane, each with a conductance of about 0.1 nmho. The estimate of pore conductance rests largely on the fact that certain subthreshold potentials occur more often than others, with a spacing between most likely potentials of about 0.15 mV (Lüttgau, 1958).Unfortunately, the overall conductance of the nerve is too high ($\sim 10^{-3}$ mho/cm^2) to allow these supposed discrete conductance changes to be observed directly under voltage clamp conditions.

However, changes of this kind have been detected in black lipid films of the kind first described by Mueller, Rudin, Tien and Wescott (1962). Several substances, when added to black lipid films, induce strongly voltage-dependent conductances. Such substances provide models which may be examples of rather general mechanisms by which voltage gating can be accomplished. Studies have been performed using gramicidin (Hladky & Haydon, 1972; Bamberg & Laüger, 1973), EIM (Bean, Shepard, Chan & Eichner, 1969; Ehrenstein, Lecar & Nossal, 1970), monazomycin (Muller & Finkelstein, 1972) and alamethicin (Mueller & Rudin, 1968; Gordon & Haydon, 1972). Alamethicin and monazomycin show the strongest voltage dependence and alamethicin has the additional advantage that its primary structure is known (Payne, Jakes & Hartley, 1970). Gramicidin, EIM and alamethicin have been shown to produce holes through black lipid films, and it is suspected that monazomycin is also a pore former.

Alamethicin exhibits a strongly voltage-dependent conductance in membranes formed from 2.5% sphingomyelin in tocopherol, chloroform, methanol, 5:3:1 (Mueller & Rudin, 1968). In membranes of this composition, addition of alamethicin to one side produces an increase in electrical conductance when voltages of either polarity are applied. The conductance increase occurs at a somewhat lower voltage when the side to which the alamethicin is added is positive. Gordon and Haydon (1972) published the first evidence that alamethicin produces discrete steps in conductance. They also stated, that the discrete steps come in "groups", but they did not provide quantitative evidence that this is the case or connect the measurements of discrete steps with the strongly voltage-dependent conductance observed at higher conductance levels. Gordon and Haydon (1972) have stated that the conductance of a discrete level is independent of membrane

thickness, is approximately proportional to electrolyte concentration and is nearly independent of voltage but shows a slight bend toward the current axis in the voltage-current curves at higher voltages.

Cherry, Chapman and Graham (1972) found that alamethicin in their membranes (lecithin-decane, 1% w/v) also induces a conductance increase independent of voltage and that the type of ion influences the rate at which the current increases with voltage.

Alamethicin has been studied also in systems other than the black lipid film. Hauser, Finer and Chapman (1970) have used high resolution NMR to study the interaction of phospholipid vesicles and alamethicin. The lipids used were egg lecithin and phosphatidyl serine. They found that the signals of both the choline groups and the hydrocarbon chains are progressively broadened by the addition of alamethicin to *sonicated* lecithin preparations. Phosphatidyl serine peaks are also broadened by the addition of alamethicin to *sonicated* vesicle preparation. They interpret these results as indicating a cooperative interaction between lipid and alamethicin. Recent results (A. Lau & S. I. Chan, 1973, *personal communication*), however, indicate that the peak broadening results from an ability of alamethicin to induce aggregation between small vesicles coupled with the fact that small vesicles exist in an inherently disordered state (Sheetz & Chan, 1972).

McMullen and Stirrup (1971) have studied the interfacial tension of alamethicin at water-hydrocarbon interfaces and performed sedimentation studies as a function of alamethicin concentration in various solvents. They found that alamethicin produces aggregates which increase in size with increasing alamethicin concentration and that the critical micellar concentration (c.m.c.) in dilute NaOH at pH 8.0 at 30 °C is 2.4 μM.

McMullen, Marlborough and Bayley (1971) have also examined the conformational properties of alamethicin by optical rotary dispersion and circular dichroism in a variety of solvents. They found no effects of monovalent cations on the spectra, but a strong dependence on the solvent used. Spectra in aqueous (10% ethanol) solutions indicated a less ordered structure than found in pure ethanol. Studies with other solvents indicated that the more hydrophobic the solvent the more ordered the conformation as revealed by these experiments. McMullen *et al.* (1971) have speculated that the conformation of alamethicin may be determined by its location in the bilayer.

In this paper, we will show how macroscopic phenomena, voltage-current curves and negative resistance in the presence of a salt gradient, for example, are related to microscopic events, events which presumably involve interactions of only a relatively few molecules. On the macroscopic level

we will present observations of voltage-current curves, time responses of current to a voltage step and time averaged fluctuations at high conductance levels. On a microscopic level we have measured, in the same system used for the macroscopic observations, discrete conductance levels of the kind reported by Gordon and Haydon (1972). We have, in addition, made quantitative measurements on the relative likelihood of given discrete levels and can show that the strongly voltage-dependent conductance must depend on the voltage dependence of the probability of formation of a single entity, which we call a "pore". Each pore can then exist in several conductance states, corresponding to the discrete levels. We also present data confirming some of the reports of Gordon and Haydon concerning discrete steps.

These results put constraints on the properties which a molecular model of alamethicin-induced conduction must have, and we present a simple model which exhibits the required properties.

Materials and Methods

Apparatus

The apparatus consisted of three basic components; the chamber, the supporting assembly and the electrical equipment. Several types of chambers were used, all had vertical septa in which the membrane-forming orifice was located. The volume of the front compartment was equal to that of the back for all chambers used. Chambers were made from two materials, polyvinyl chloride and Teflon; both types of chambers gave identical electrical results. Membranes were formed in two ways. First, we used a Pasteur pipette fitted with a rubber bulb. By squeezing the bulb, a small air bubble could be formed at the tip of the pipette. The air bubble could then be used as a "brush" to spread a membrane using membrane-forming solution already introduced. Second, a feeder hole (0.65 mm dia.) was drilled through the chamber septum to the membrane hole. This passage was connected externally to a Hamilton 10-μliter syringe. Membrane solution was introduced into the hole, and by moving the syringe plunger an appropriate distance, a membrane of the desired area could be formed.

To provide mechanical isolation, the membrane chamber was mounted on a cylindrical lead block weighing 25 kg. The block was in turn mounted on an inflated minibike innertube. This arrangement provided vibration isolation comparable to that of much more elaborate systems. The lead block was drilled out to provide room for the introduction of a motor and magnets for driving Teflon-coated stirring bars in both chambers. Because of electrical noise, the solutions were not stirred during low level measurements; however, they were thoroughly stirred before such measurements and also at higher current levels. The current amplifier was mounted in a shielded enclosure attached to the lead block. The membrane cell and the current-measuring amplifier thus formed parts of the same essentially rigid mechanical assembly. This design eliminated microphonics resulting from positional changes of the cell relative to the amplifier.

The electrical apparatus consisted of a basic voltage supply amplifier, an electrometer amplifier to measure the voltage, and a current amplifier for current measurement. This equipment followed the design of Huebner and Bruner (1972), but the current-

measuring amplifier was specially guarded to allow operation at high frequencies and with high feedback resistors (Eisenberg, 1972). Because of the guarding technique, wiring capacitance provided sufficient capacity to just stablize the amplifier against oscillation with a feedback resistor of $10^8\ \Omega$. This combination resulted in a system capable of responding to a step current change of 10^{-11} amps in 1 msec. For higher currents the response time was proportionally shorter. Four electrodes were used. One pair provided (and measured) the current, the other measured the voltage using the high impedance differential electrometer amplifier. Electrodes made of chlorided silver wire (Ag–AgCl) were used for most measurements, but for some measurements, in particular those involving asymmetric chloride concentrations, commercial calomel electrodes (Beckman Instruments) with KCl–Agar bridges were used.

The basic voltage supply amplifier was provided with an internal ramp which was used for taking current *vs.* voltage curves. The positive and negative excursions of the ramp were independently variable from 0.0 to +1 V or −1 V, respectively. The rate of change of voltage with respect to time was variable in steps from 0.45 mV/sec to 135 mV/sec. The sweep could be interrupted at any time and the voltage clamped at any desired value. The voltage amplifier was also commanded by a pulse generator, which operated either in a continuous mode or a single cycle mode (Eisenberg, 1972). In the continuous mode, the voltage oscillated between two preset values. The time spent at each value was adjustable independently from 0.1 msec to 20 sec. In the single cycle mode the voltage was set initially at a third independently adjustable level and, on command, cycled once between the two preset levels, each with a preset duration as before, and then returned to the starting point.

To measure current amplitude distributions, special logic circuitry designed to operate in conjunction with a Princeton Applied Research waveform eductor, Model TDH-9 (Princeton, New Jersey) was used (Eisenberg, 1972). The circuitry, in essence, converts the TDH-9 to a 100-channel analogue memory with an adjustable sampling rate. The current is sampled for 1 μsec and one count placed in the channel corresponding to the value of the current. The proportionality between channel number and current can be varied over a wide range. The current histogram can be displayed on an oscilloscope as it builds up. Fig. 1 shows a block diagram of the measuring system and the relationships of the various components.

A Mosely X-Y recorder was used to record both current *vs.* voltage curves and histograms generated by the waveform eductor.

Membrane Materials

Chromatographically pure phosphatidyl ethanolamine (PE) from bacterial sources, was purchased from Analabs Inc. (North Haven, Connecticut), Supelco Inc. (Bellefonte, Pennsylvania), Calbiochem (La Jolla, California) and General Bio-Chemicals (Chagrin Falls, Ohio). We used primarily the Supelco lipid (catalog No. 04-6040). Its fatty acid composition was not analyzed by us, but the suppliers claim it is composed of 80% saturated hydrocarbon chains of which a third have cyclopropyl branching. The specific capacitance of membranes formed from the Supelco lipid was 0.53 $\mu F/cm^2$. We used *n*-decane from Eastman (Rochester, New York) and Aldrich Chemical Co. (Milwaukee, Wisconsin). The salts, of ultra-pure quality, were purchased from Mann Research Lab. (Orangeburg, New York) and Alfa Inorganics, Ventron (Beverly, Massachusetts). Glycerol, analytical reagent grade, was obtained from Malinckrodt (St. Louis, Missouri).

Alamethicin was kindly provided by Dr. G. B. Whitefield of Upjohn Co. (Kalamazoo, Michigan) and was used without further purification. Payne *et al.* (1970), in their determination of the primary structure concluded that this material was probably

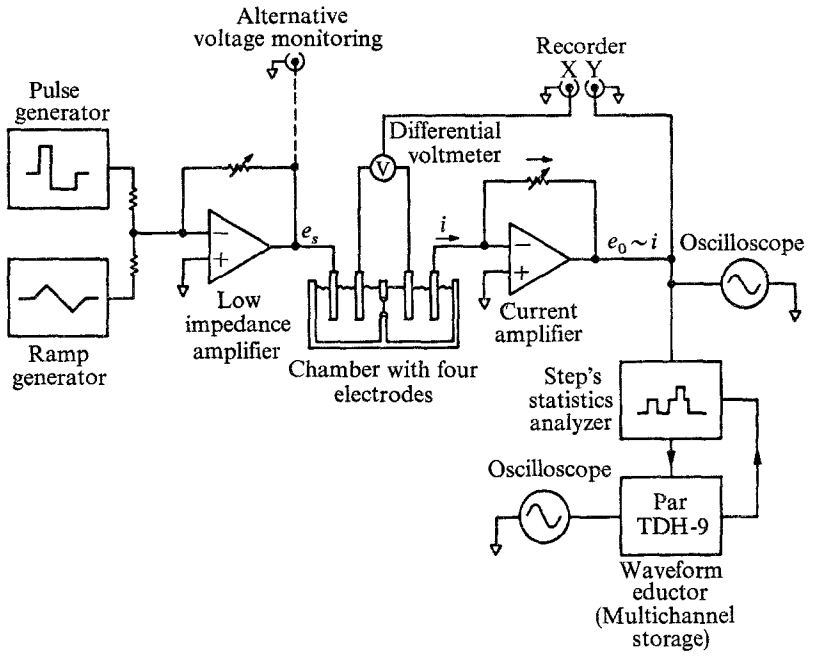

Fig. 1. Block diagram of the electrical measuring system

composed of two or more fractions which only differed in their relative contents of alanine and α-aminoisobutiric acid by one amino acid.

Distilled and bottled Arrowhead Puritas (Los Angeles, California) water was passed through a "Deeminizer" demineralizer from Crystalab (Hartford, Connecticut). The conductivity was typically 2×10^{-7} mho/cm.

Membrane-forming solution was made by dissolving PE in *n*-decane at 1.25% weight/volume at 65 °C for 1 min, under nitrogen; it was stored at −20 °C and warmed to room temperature for use.

Stock solutions of salts were prepared at high concentration and diluted as required. Alamethicin stock solutions in water were made to 10^{-3}, 10^{-4} and 10^{-5} g/ml and were stored at 2 °C. The 10^{-3} g/ml solution of alamethicin in water was prepared by stirring the alamethicin-water mixture continuously overnight at 2 °C. It should be noted that almost all of our experiments were carried out with alamethicin concentrations below the c.m.c. of 2.4 μM as measured by McMullen and Stirrup (1971).

Results

Voltage-Current Curves at High Levels

For the sake of clarity, we will distinguish between the *front* chamber and the *back* chamber of the measurement cell. We will consider a *positive current* to be one in which cations flow from front to back and a *positive*

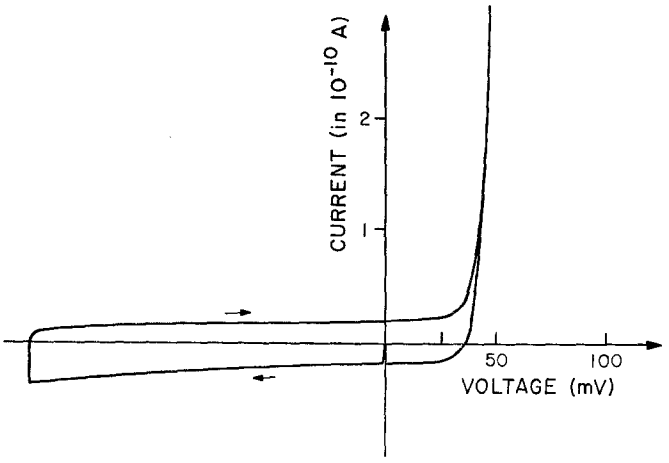

Fig. 2. Voltage-current curve of a PE-decane membrane 8.5×10^{-3} cm^2 in area, with 0.1 M NaCl front and back and 3×10^{-6} g/ml alamethicin *front only*. $dV/dt = 4.5$ mV/sec. Arrows show direction of voltage sweep

voltage to be one where the potential of the front chamber is more positive than that of the back. Our results differ from those of Mueller and Rudin (1968) and of other authors (Cherry *et al.*, 1972; Gordon & Haydon, 1972) in two significant respects. First, when alamethicin is added to only the front chamber, a conductance increase is observed only when the voltage is positive, and no increase is seen for a negative polarity. Second, we observe no measurable voltage-independent conductance increase above the conductance level of the bare membrane. Fig. 2 shows a typical current *vs.* voltage (I–V) curve measured at a voltage sweep rate of 4.5 mV/sec. The separation of the traces in the two different directions (indicated by arrows) is due to the capacitive charging current and is given by $i_c = C(dV/dt)$. Conductance below about 25 mV is indistinguishable from that of black lipid film without additive. (Bare membrane conductivity is 1.0×10^{-8} mho/cm^2.) The alamethicin concentration is 3×10^{-6} g/ml. If identical amounts of alamethicin are added to both sides, the I–V curve is symmetrical. Addition of alamethicin to one side of the membrane produces effects which are independent of those produced by adding alamethicin to the other side (with the condition that the membrane has not broken). This is in marked contrast to the results of other workers, and the difference is probably due to the choice of membrane lipids, in particular to our using a lipid with saturated fatty acids. In experiments using dioleoyl-lecithin/decane membranes (1.2% w/v), our results were similar to those of other workers. This

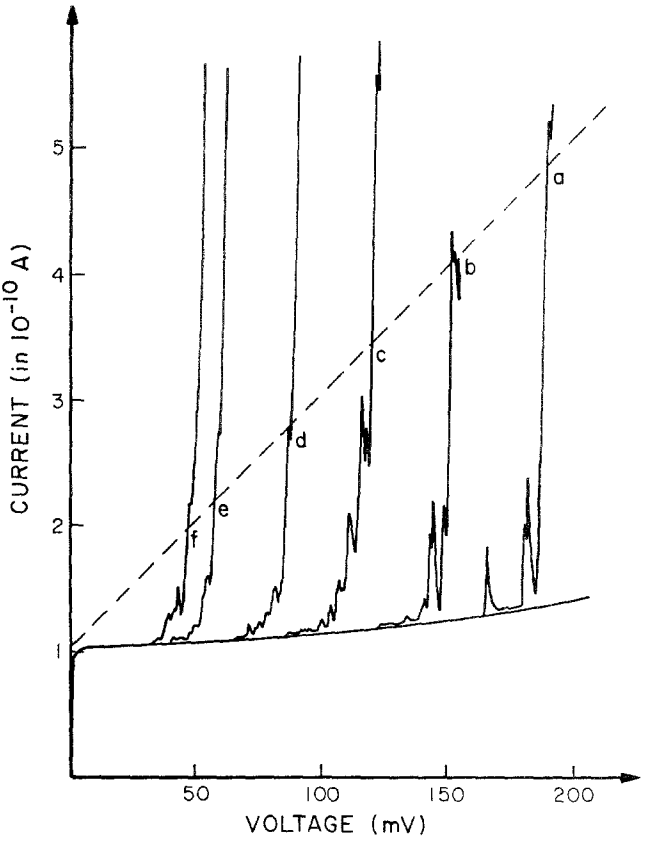

Fig. 3. Family of voltage-current curves in symmetric 0.1 M NaCl with different alamethicin concentrations (*a*) 5×10^{-8} g/ml, (*b*) 10^{-7} g/ml, (*c*) 2×10^{-7} g/ml, (*d*) 5×10^{-7} g/ml, (*e*) 10^{-6} g/ml and (*f*) 1.5×10^{-6} g/ml ($dV/dt=12$ mV/sec). The baseline current is the sum of the capacitive charging current and the current through the bare membrane. The dashed line is the fixed conductance G_c

indicates that the presence of unsaturated hydrocarbons in the membrane lipids influences the ease with which alamethicin diffuses across the membrane.[1]

1 Preliminary measurements (Johnson, 1973) show that the addition of alamethicin to one side only of a synthetic di-isostearoyl-phosphatidylcholine/decane (1 % w/v) black lipid membrane, induces asymmetric I–V curves similar to those obtained with the bacterial PE-decane membranes in our experiments. This synthetic lecithin has fully saturated hydrocarbon chains, in contrast to egg lecithin, which is rich in unsaturated fatty acyl groups. When the egg lecithin is used to form the membranes, there is a much lesser degree of asymmetry in the I–V characteristics, and a zero-volt conductance, independent of voltage, is observed, (Mueller & Rudin, 1968; Cherry *et al.*, 1972). One infers from these observations that saturated hydrocarbon chains allow little diffusion of alamethicin across the membrane, ("little" relative to the rate of diffusion across the aqueous and unstirred layers).

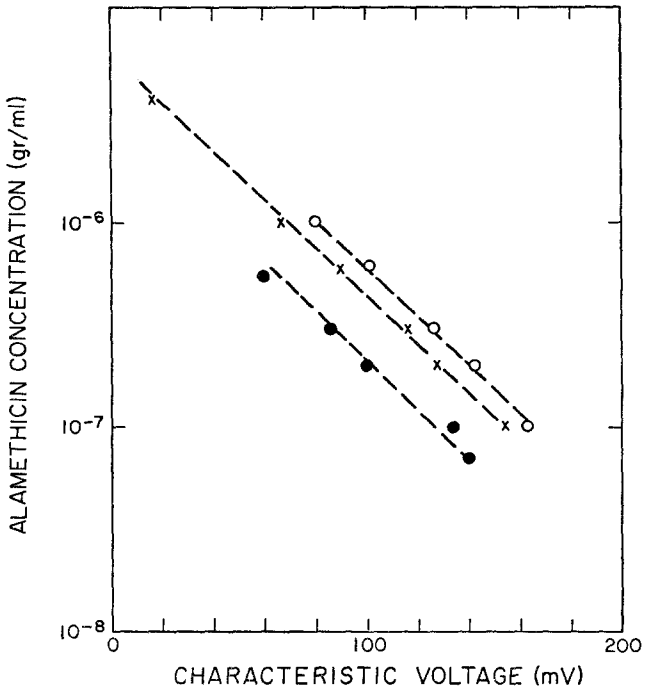

Fig. 4. Characteristic voltage as a function of alamethicin concentration in 0.1 M solutions of different chloride salts. ×-×-× NaCl, -o-o- KCl, -•-•-• $BaCl_2$

In this paper we will consider *only* the case of bacterial PE-decane membranes because the effects appear simpler than in the case of other lipids. Nonetheless we should emphasize that lipid and fatty acid differences may be quite important in understanding the detailed molecular mechanism by which alamethicin produces its effects on membranes.

Current *vs.* voltage curves of the kind shown in Fig. 2 can be used to study the effect of varying alamethicin concentration, salt concentration and salt type. Fig. 3 shows a series of I–V curves obtained at different alamethicin concentrations. These curves are similar except for a shift along the voltage axis. To measure the dependence of this shift on alamethicin and salt concentrations we specify a *fixed* conductance G_c and define a characteristic voltage V_c as the voltage at which this conductance is reached. We have chosen G_c to be approximately one order of magnitude larger than bare membrane conductance. V_c decreases with increasing alamethicin concentration as shown in Fig. 4. The form of the dependence is logarithmic and can be written as

$$V_c = -V_a \ln \frac{C_a}{C_0} \tag{1}$$

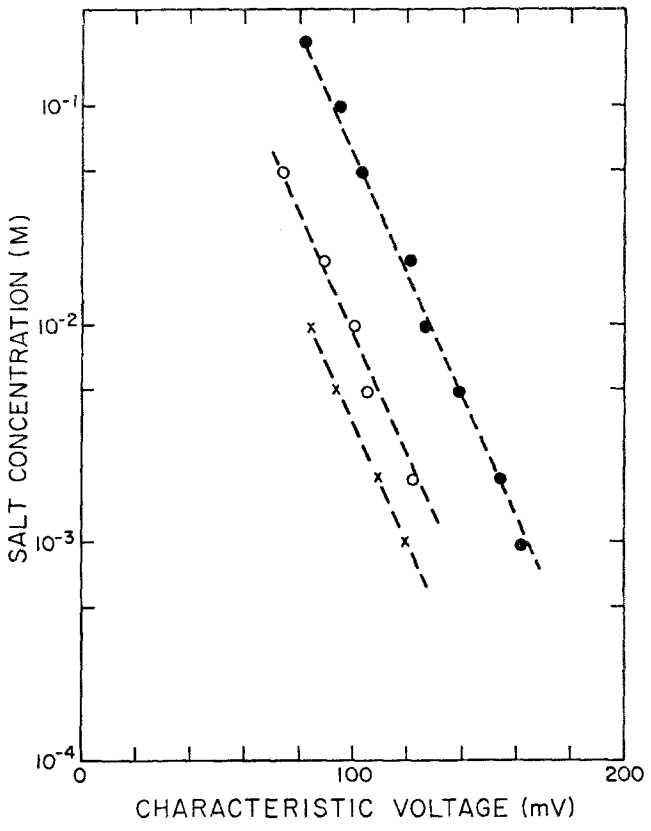

Fig. 5. Characteristic voltage as a function of salt concentration at constant alamethicin concentration; -x-x-, NaCl with 2×10^{-6} g/ml alamethicin; -o-o-, KCl with 10^{-6} g/ml alamethicin; -•-•-, $CaCl_2$ with 2×10^{-7} g/ml alamethicin

where C_a is the alamethicin concentration and C_0 some reference concentration which depends on membrane area, salt concentration and other factors. V_a, the slope of the logarithmic dependence, is 36 ± 3 mV for the three chloride salts studied.

The characteristic voltage also decreases when the salt concentration is raised. The dependence is again logarithmic with C_0' as some reference concentration:

$$V_c = -V_{salt} \ln \frac{C_{salt}}{C_0'}. \tag{2}$$

Fig. 5 shows results for NaCl, KCl and $CaCl_2$, in the presence of fixed alamethicin concentrations. V_{salt} has the same value, 16 ± 1.5 mV, for the three salts studied.

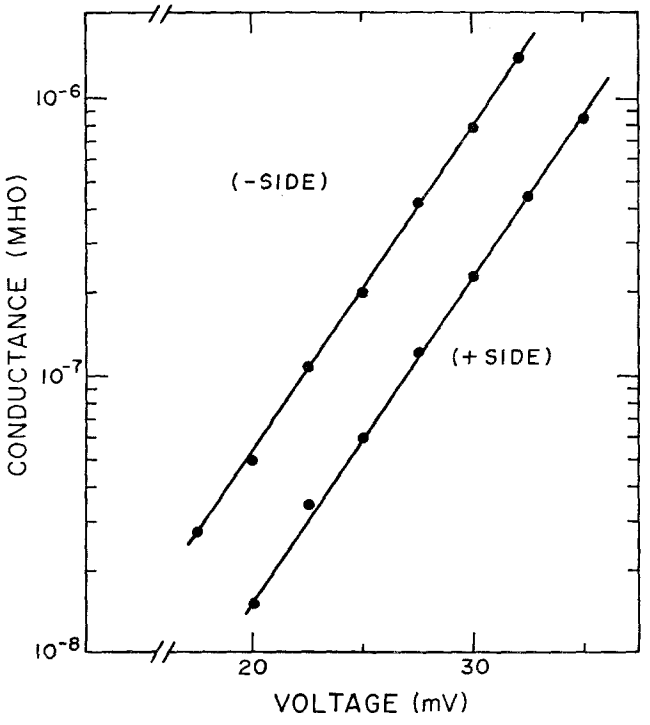

Fig. 6. Conductance of a PE-decane membrane in 1 M NaCl with 10^{-6} g/ml alamethicin on both sides plotted on a semi-logarithmic scale to show the exponential dependence of the conductance on voltage

We can combine Eqs. (1) and (2) to give the dependence of V_c on both salt concentration and alamethicin concentration:

$$V_c = V_i - V_{\text{salt}} \ln C_{\text{salt}} - V_a \ln C_a \tag{3}$$

where V_i is the characteristic voltage at unit concentrations of alamethicin (in g/ml) and of salt (M). V_i depends on salt type and on membrane area.

Fig. 6 shows a conductance-voltage curve in 1 M NaCl with approximately 10^{-6} g/ml alamethicin on both sides, plotted in semilogarithmic fashion. The curves do not coincide because the concentrations of alamethicin on the two sides are not precisely equal, but the voltage dependence of both branches is exponential and the conductance is seen to have the following form:

$$G = G_0 e^{V/V_0}. \tag{4}$$

V_0 is 3.94 ± 0.35 mV, when measured in aqueous solutions of either NaCl, KCl or $CaCl_2$.

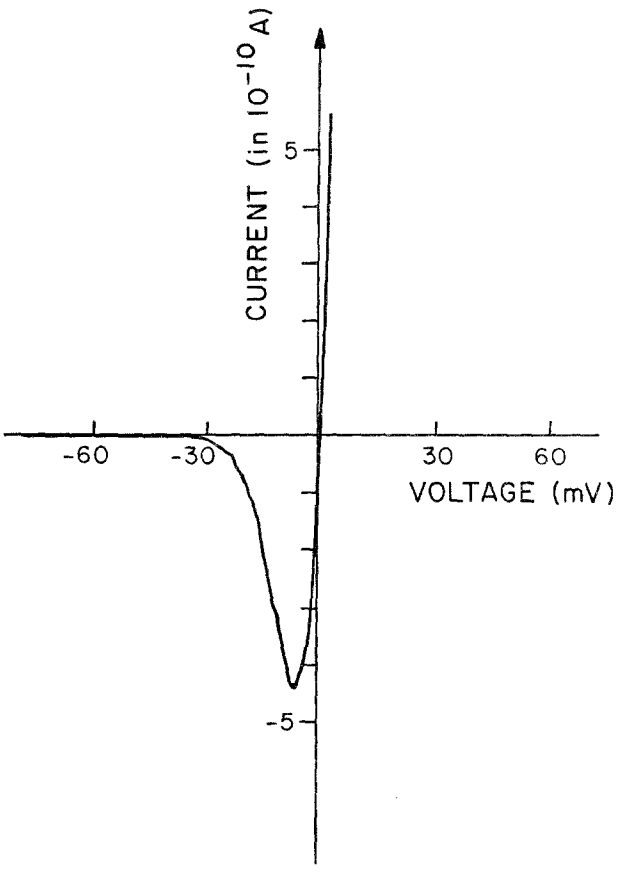

Fig. 7. Voltage-current curve of a PE-decane membrane 0.5×10^{-2} cm^2 in area with 1.0 M NaCl on both sides and 10^{-5} g/ml alamethicin added to the front only

The slope V_0 of the exponential voltage dependence of the conductance is independent of the alamethicin concentration. This property of the system is responsible for many of the excitability phenomena reported (Mueller & Rudin, 1968; Boheim, 1972). If sufficient alamethicin is added to only one side of the membrane, V_c becomes negative, and an I–V curve is obtained which exhibits a negative resistance with zero current at zero voltage. Fig. 7 shows a negative resistance curve measured with 10^{-5} g/ml alamethicin added to the front chamber and 1 M NaCl both front and back. The conductance for both positive and negative voltages shows the same exponential dependence as that given in Eq. (4) with $V_0 = 4.2$ mV. In this case we observe a zero voltage conductance far above that of the bare membrane. This conductance is strongly voltage dependent in contrast to the zero volt conductance of Cherry *et al.* (1972).

Perfusion and Dilution Experiments

In this section we show that alamethicin adsorbs reversibly to the membrane surface, and that little of the alamethicin added to the aqueous solution is lost by adsorption to the various interfaces.

We show in two ways that alamethicin appears to adsorb reversibly to the membrane surface. First, we add alamethicin to only the front chamber of an already black film. There is a characteristic voltage in the positive direction only. The film is subsequently broken and respread within two seconds. The positive V_c remains constant at the same value of the first film, but there is now a characteristic voltage in the negative direction as well, which increases linearly with time. For a chamber volume of 8 ml (each side) and a membrane area of 2.1×10^{-3} cm^2, we find that the negative characteristic voltage changes linearly with time at a rate of 8.5 mV/min. Since, in equilibrium, the characteristic voltage is proportional to the logarithm of the concentration of alamethicin in the aqueous phase [*cf.* Eq. (1)], we conclude that the alamethicin concentration on the back side of the membrane decays exponentially toward a very low final value. The half life of alamethicin at the membrane, calculated from Eq. (1), is 2.9 min.

Similar results are obtained from perfusion experiments. A fixed amount of alamethicin is added to the front chamber. The front chamber solution is then exchanged at a constant rate with a solution free of alamethicin by a perfusion pump. Differentiation of Eq. (1) with respect to time shows that V_a may be calculated from the assumed value of the alamethicin concentration as a function of time (determined by the perfusion apparatus) and the measured value of dV_c/dt. This method is not very accurate, but the value of V_a obtained in this manner is in fair agreement with that obtained by the experiments described previously. The characteristic voltage does increase *linearly with time* indicating an *exponential decrease of alamethicin concentration.*

It might be argued that these results could be explained by alamethicin adsorbing to and desorbing from the thick border region, the air-water interface or the Teflon-water interface. These arguments can be ruled out on the following grounds. First, by the nature of the measurement (a change of V_c) we are observing a phenomenon localized in the membrane. We can therefore ask how long it would take for these interfaces to arrive at equilibrium with the membrane, solely through the mechanism of lateral diffusion of alamethicin along the surface. If we assume a value of 10^{-8} cm^2/sec for the lateral diffusion constant (Bamberg & Laüger, 1973) and a diameter of 10^{-2} cm for the membrane, the time for diffusion would then be of the order of 10^4 sec ($t \sim x^2/D$). The effects we observe clearly take place on a

time scale very short compared to 10^4 sec. In fact, the diffusion constant would have to be three orders of magnitude larger for the diffusion time scale to be comparable to that observed. Clearly then the major factor determining the concentration of alamethicin in the membrane is the interaction between the membrane and the bulk aqueous phase (across the unstirred layer). One potential problem is that alamethicin adsorption on various interfaces might reduce the concentration in the aqueous phase. The following experiments were performed to show that alamethicin is not adsorbed sufficiently to alter the bulk concentration appreciably. A membrane was formed in the usual way with 10^{-7} g/ml alamethicin, 1.0 M NaCl, both sides. The solution was pipetted out to another chamber and a membrane formed in the new chamber. The original chamber was refilled with new salt solution and a new membrane formed. The measured values of V_c were then used to calculate the alamethicin concentration in both. The calculated concentration in the new chamber was 1.3×10^{-7} g/ml $\pm 0.34 \times 10^{-7}$ g/ml and that in the old chamber $1.24 \pm 0.11 \times 10^{-8}$ g/ml. It should be noted that some solution was left in the old chamber and no effort was made to clean off lipid-forming solution from the first experiment.

These experiments show that the aqueous concentration of alamethicin is not appreciably reduced by adsorption to the walls of the chamber and that no more than 12% of the alamethicin can possibly stick to the walls, form an air-water interface, or be adsorbed in the lipid phase. This estimate is high since the original chamber was not rinsed out and a small amount of solution remained, accounting for perhaps half of the above result.

Discrete Current Levels

We have studied low level current fluctuations, similar to those reported by Gordon and Haydon (1972). Fluctuations occur reasonably often near the characteristic voltage and can then be resolved as spikes in the I–V curve (*cf.* Fig. 3). A more effective method for observing these fluctuations is to clamp the voltage at a given value and observe the fluctuations in current as a function of time. Fig. 8*a* shows an oscilloscope trace of such a record taken at a voltage of 145 mV. Inspection reveals that the current takes five discrete levels (above the bare membrane level), some with a greater probability than others. Fig. 8*b* shows some of the fluctuations at a faster sweep rate to clarify the time response of the measuring system.

Since the relative probability of the conductance levels (or conductance states) might be the strongly voltage-dependent function we are searching for, it is important to quantify the relative probabilities of the various levels. Fig. 9 shows a distribution of the number of occurrences versus current. The

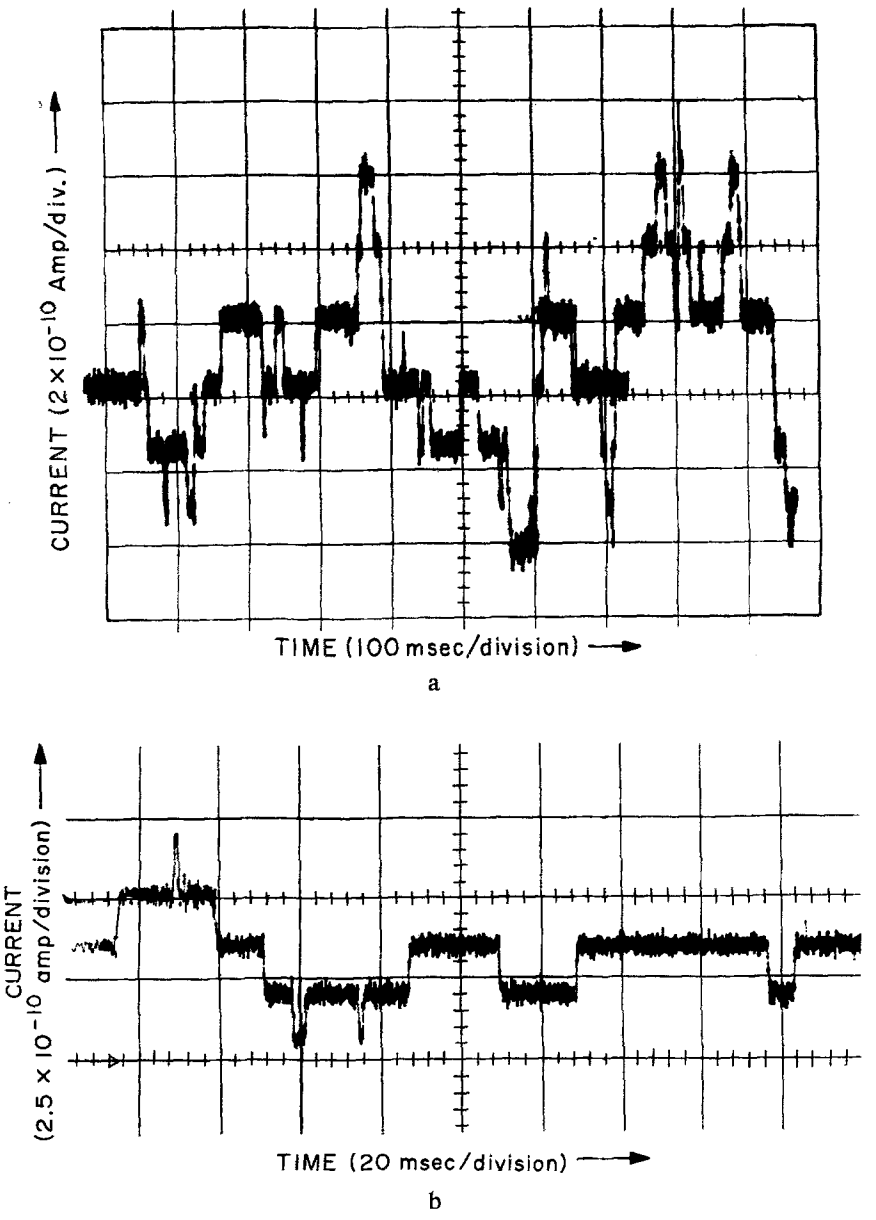

Fig. 8. (*a*) Oscilloscope trace showing current as a function of time for a PE-decane membrane in 1 M NaCl and 10^{-7} g/ml alamethicin at 145 mV. The lowest level corresponds to the current level of the unmodified membrane. (*b*) Same but at a faster time sweep

peak corresponding to bare membrane current is much larger than the others, and is drawn on a reduced scale. Note that five different levels are resolved and that the second and third levels are the most likely. The

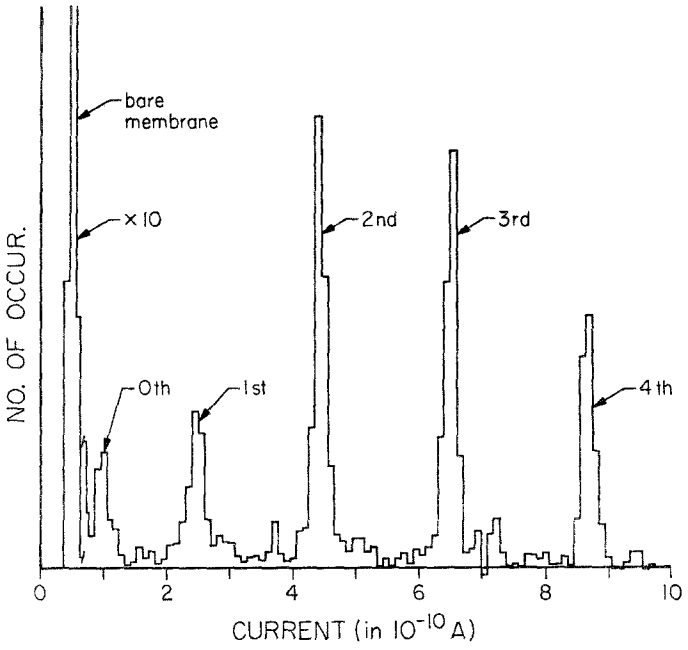

Fig. 9. Current distribution for a 0.4×10^{-2} cm^2 PE-decane membrane in 1 M NaCl and 3×10^{-8} g/ml alamethicin. The voltage is 207 mV

differences between consecutive peaks are not equal. In 1.0 M NaCl, the successive differences in units of 10^{-10} mho are: 2.3 (bare membrane to 0th level), 7.1 (0th to 1st level), 9.4 (1st to 2nd level), 10.0 (2nd to 3rd level) and 10.5 (3rd to 4th level). Thus, no level is an accurate integral multiple of a lower level.

We can use these distributions and oscilloscope traces to characterize the levels as functions of voltage, salt concentration and type of salt used. We consider first the voltage-current curves of the single levels.

Current distributions as well as oscilloscope tracings were taken for 1.0 M solutions of various chloride salts. Individual current steps can be resolved only at low conductance. Thus, alamethicin concentration was increased stepwise to lower the voltage at which fluctuations were resolved. Overlap between the conductances measured at different alamethicin concentrations showed that the conductance levels do not depend on the alamethicin concentration. Fig. 10 shows the current-voltage characteristics of the resolvable conductance levels for 1.0 M NaCl aqueous solutions, for many different alamethicin concentrations, and several different films (all PE-decane). The reproducibility of the conductance data is good, and the discrete conductance levels are nearly, but not perfectly, ohmic. The conductance of a given level increases slightly with voltage. Fig. 11 shows the

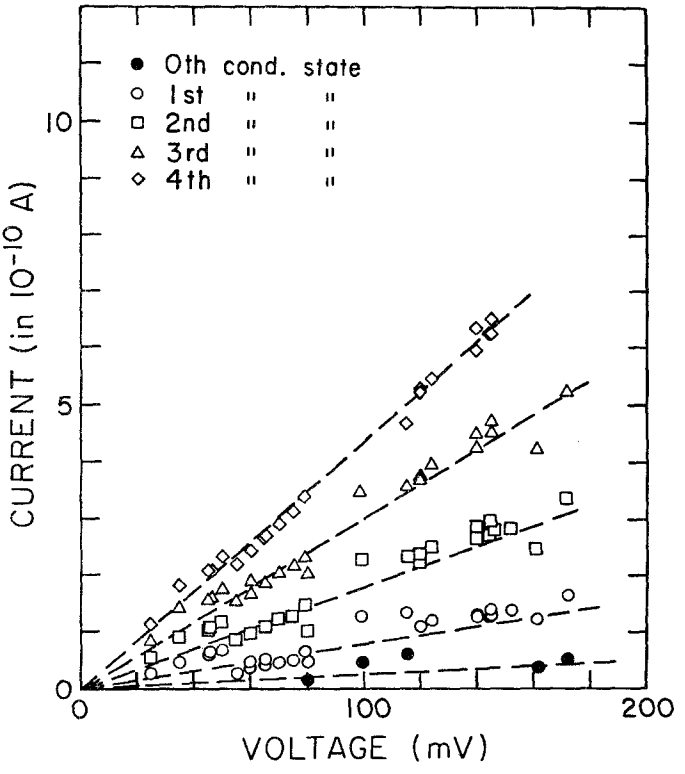

Fig. 10. The voltage-current curve of the discrete levels in 1 M NaCl. The data represent a number of different membranes and alamethicin concentrations. The data in Fig. 11 are included

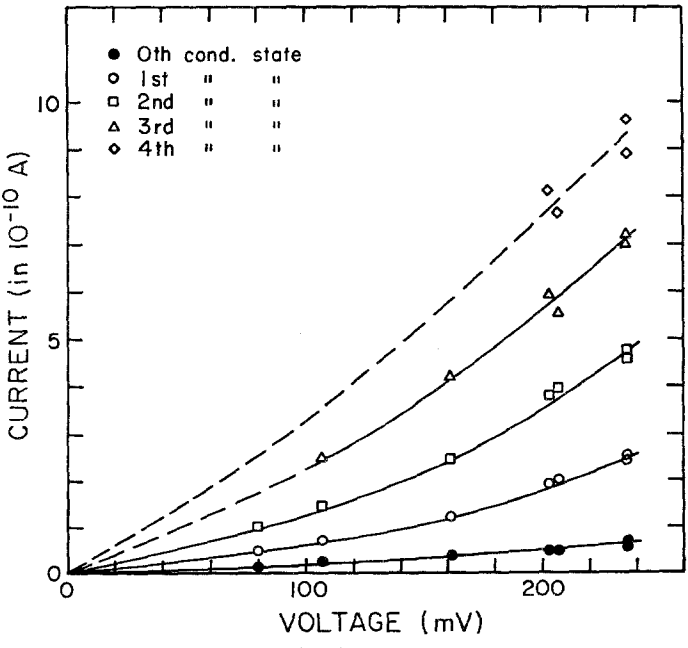

Fig. 11. The voltage-current curve of the discrete levels in 1 M NaCl taken on a single membrane up to 240 mV

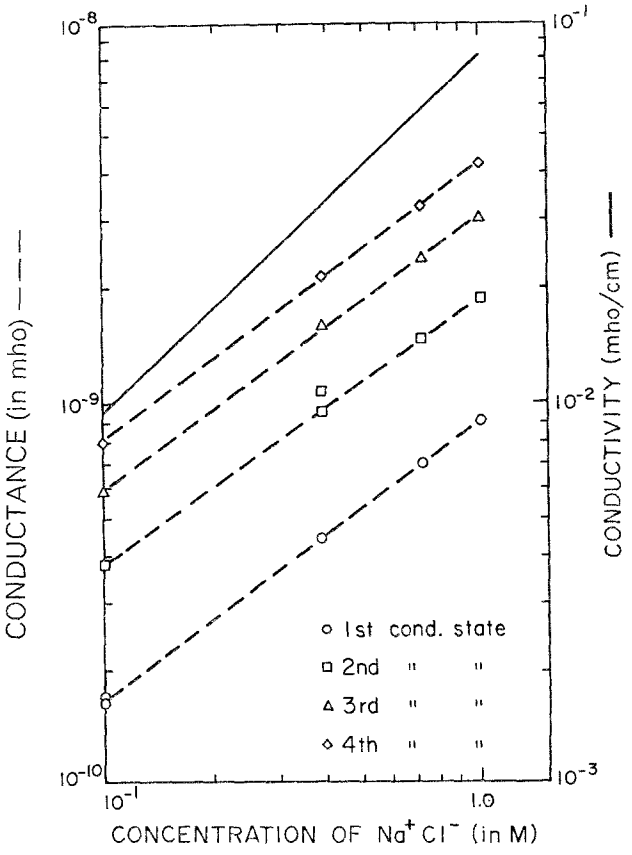

Fig. 12. Conductance of the discrete levels as a function of salt concentration. The dependence of bulk conductivity on concentration is shown by the solid line for comparison. Conductance is shown on the left-hand scale, conductivity on the right-hand. The zeroth level is omitted because of the difficulty of accurate measurement at low concentrations

nonlinearity for a single experiment on one film. It covers a larger voltage range than Fig. 10. The data of Fig. 11 are included in Fig. 10.

To measure the dependence on salt concentration of the conductance of any discrete level, current distributions which resolved discrete steps were taken for PE-decane black membranes in symmetric solutions of sodium chloride at concentrations ranging from 0.1 M to 1.0 M. The conductances of the first to fourth levels are plotted against sodium chloride concentrations on a log-log graph (Fig. 12), and the conductivity of bulk sodium chloride in the same concentration range is shown for comparison. The slopes for all four conductance levels are equal but slightly smaller than those of the bulk solutions.

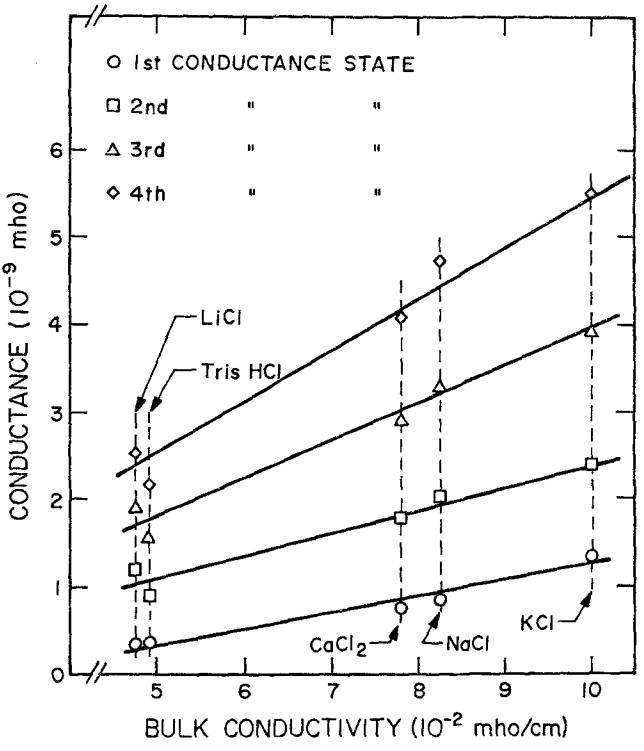

Fig. 13. Conductance of discrete levels at 1 M salt concentration plotted against the conductivity of the corresponding 1 M bulk solution for several ions. Note that the discrete level conductance is proportional to the bulk conductivity of the salt

Different cations were compared by taking current distributions of discrete steps for 1.0 M solutions of various chloride salts on both sides and with 2×10^{-8} g/ml alamethicin added to the front. The results are plotted in Fig. 13 and show that the conductances of the discrete levels depend in the same way on the cationic species as do the bulk conductances.

We have also measured the dependence of discrete conductance changes on the viscosity of a 1.0 M NaCl solution, by adding various concentrations of glycerol. Fig. 14 shows that conductances of the first to fourth level are inversely proportional to the viscosity of the glycerol solutions at 23 °C. The conductivity of 1.0 M NaCl solution as a function of viscosity is plotted on the same graph for comparison. Again the bulk solution and the conductance of the discrete levels correlate rather well.

The conductance of the discrete levels is in the range 0.1 to 1 nmho for salt concentrations in the hundred millimolar range. Such a conductance is too large to be accounted for by a discrete carrier diffusing back and forth

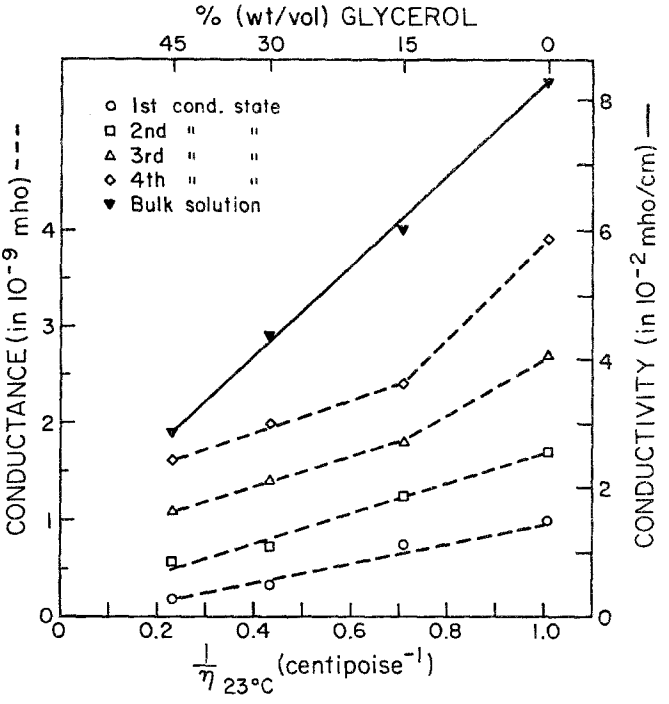

Fig. 14. Conductance of first to fourth conductance levels of a pore in 1.0 M NaCl solutions as a function of glycerol concentration and viscosity (left-hand scale). Bulk solution conductivities are shown for comparison (right-hand scale)

across the membrane. The carrier flux required at 100 mV would be about 10^8 round trips/sec, which would in turn imply a diffusion constant of 10^{-5} cm^2/sec, a value comparable to the diffusion constant of a small ion such as potassium in water. This calculation, combined with the behavior of the conductance levels as functions of voltage, salt concentration and solution viscosity, leads to the conclusion that alamethicin induces pores in the membrane and that the "opening" and "closing" of these pores gives rise to the induced conductance. The pore, once formed, fluctuates in the 10 to 1,000 Hz range between about five conductance levels.[2]

Pores as Statistically Independent Units

Since the conductance of the discrete levels of a pore does not increase appreciably with voltage, the exponential dependence of current on voltage

2 This rate of fluctuation is a qualitative estimate. We are currently investigating fluctuation rates quantitatively. It appears that transitions between levels of a pore occur with equal probability per unit time, and that the probability of a transition is a function of the level.

requires that the number of discrete levels increase with voltage (either levels per pore, or number of pores), and that the current occupy the higher levels for a proportionately greater time. This increase in number of discrete levels and the distribution over these levels (in the time domain), will be the subject of this section.

Consider the distribution, measured at 207 mV, shown in Fig. 9. A convenient way of checking the voltage dependence of such a distribution is to measure its mean as a function of voltage. The mean is defined as

$$\bar{\gamma} = \frac{\sum_{i=0}^{4} g_i A_i}{\sum_{i=0}^{4} A_i} \tag{5}$$

where $\bar{\gamma}$ is the mean conductance, g_i the conductance of the i^{th} level and A_i the probability of the i^{th} level. Experimentally, we find the value of $\bar{\gamma}$ is at most a very weak function of voltage. Thus, a shift of the distribution among the conductance levels of the individual pores contributes very little to the voltage dependence of the conductance. On the other hand, the likelihood that a pore is formed is a strong function of voltage. In fact, if the voltage at which the distribution of Fig. 9 was measured had been lowered, the only effect would have been to increase the height of the peak corresponding to bare membrane conductance relative to the heights of the other peaks. Insofar as we have been able to measure the voltage dependence of this peak ratio, we find the results consistent with the exponential increase of the *time averaged* conductance with voltage. We have not, however, been able to obtain an accurate value of the exponential slope by this method because of the difficulty of keeping a single membrane stable for a sufficiently long time to make adequate measurements.

If the voltage is sufficiently increased, the bare membrane peak becomes very much reduced and one obtains distributions such as the one shown in Fig. 15. Here the original four or five peaks are still resolved, but in addition, new peaks have appeared. The distribution can be explained if it is assumed that during the period of the sampling two pores existed simultaneously part of the time. The positions of the new levels are indeed sums of the positions of the levels of a single pore. The lines in the lower half of the figure are labeled according to their parentage; $c_{1,3}$, for example, means that the conductance is given by $g_1 + g_3$; $c_{3,3,3}$ means that the conductance is $g_3 + g_3 + g_3$.

The relative amplitudes are also as predicted. We have calculated the relative amplitudes assuming that the pores fluctuate independently, and

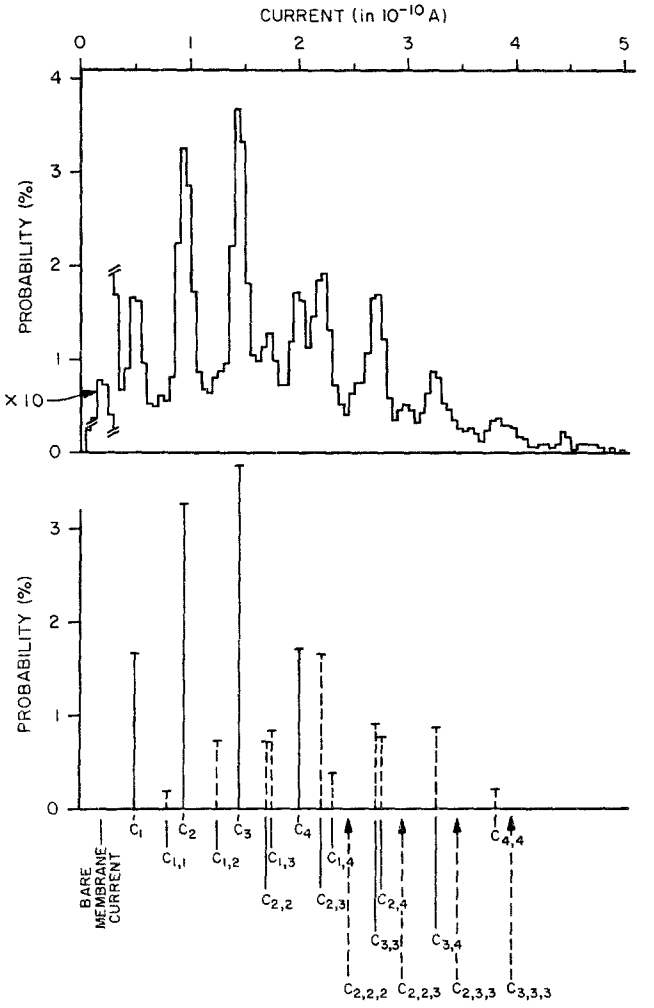

Fig. 15. Current distribution in 1.0 M NaCl and 30% glycerol. The upper part of the figure shows the experimental distribution and the lower part shows the analysis assuming that two pores exist simultaneously. The calculated positions and heights are shown as dashed lines. $C_{i,j}$ is the peak obtained from the simultaneous occurrence of one pore in the i^{th} state and another in the j^{th} state. The positions of a few triplets are shown

used the measured positions of the single pore peaks to calculate the positions of peaks attributed to more than one pore. The agreement with the observed distribution is excellent. Fig. 15 shows a distribution taken in 30% w/v aqueous glycerol solution. Similar distributions can be obtained for a wide range of alamethicin and salt concentrations. The new peaks always accord with the simultaneous existence of several pores and are consistent with the amplitude predictions.

We conclude that as the voltage increases, the average number of pores increases, but the conductance levels of each pore fluctuate independently.

The Formation of Pores: Kinetics

In the previous sections we showed that alamethicin interacts with PE-decane black membranes to form pores exhibiting discrete conductance levels. We also showed that the conductance levels of a single pore are nearly voltage independent, and that the principal voltage effect is, therefore, on the formation and disappearance of the pores. We now turn to the study of pore formation. The current response to voltage steps was monitored as a function of time. Fig. 16 shows a series of experiments (using a single membrane) with 1.0 M NaCl concentration in the aqueous phases. The magnitude of the voltage step was successively increased. After the onset of each voltage step, the current increases for a few seconds to reach a final stage where it fluctuates around a mean value.

The time course of the current is well fit by a first order kinetic equation:

$$\frac{di}{dt}=M-L\cdot i. \tag{6}$$

We attribute the change in current to the formation of pores and rewrite Eq. (6) in terms of the mean number of open pores $\bar{n}$ as

$$\frac{d\bar{n}}{dt}=\mu-\lambda\bar{n} \tag{7}$$

where μ is the rate of pore formation and λ the rate of pore decay. If there are no pores open at $t=0$,

$$\bar{n}=\frac{\mu}{\lambda}(1-e^{-\lambda t}). \tag{8}$$

At long times, $\bar{n}$ is just μ/λ. The probability that n pores are open at any given time will then be given by a Poisson distribution:

$$\rho_n=\frac{\bar{n}^n e^{-\bar{n}}}{n!}. \tag{9}$$

μ and λ are functions of voltage and experimental values derived from the voltage pulse data are plotted in Fig. 17. We should emphasize that the Poisson distribution arises from consideration of μ and λ as probabilities

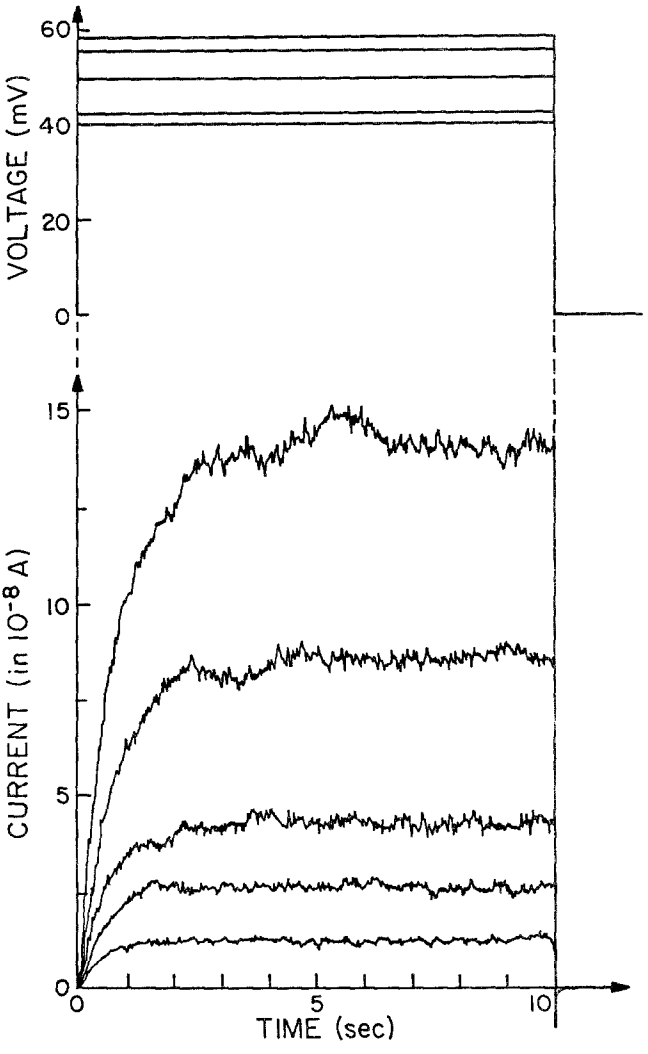

Fig. 16. The response of the alamethicin-induced current to a voltage in 1.0 M NaCl and 5×10^{-7} g/ml alamethicin. *Top:* voltage pulses of 10-sec duration and amplitude 40, 42, 50, 55 and 58 mV. *Bottom:* Current trace on X-Y recorder with 1/50 sec response

per unit time of formation and disappearance, respectively. Their functional forms are

$$\mu=\mu_0 e^{V/V_\mu}$$

and

$$\lambda=\lambda_0 e^{-V/V_\lambda} \tag{10}$$

where $V_\mu=6.7$ mV and $V_\lambda=9.6$ mV. At a concentration of alamethicin of 6.5×10^{-7} g/ml, μ_0 is on the order of 10^3 pores/(sec cm^2) and λ_0 is on the

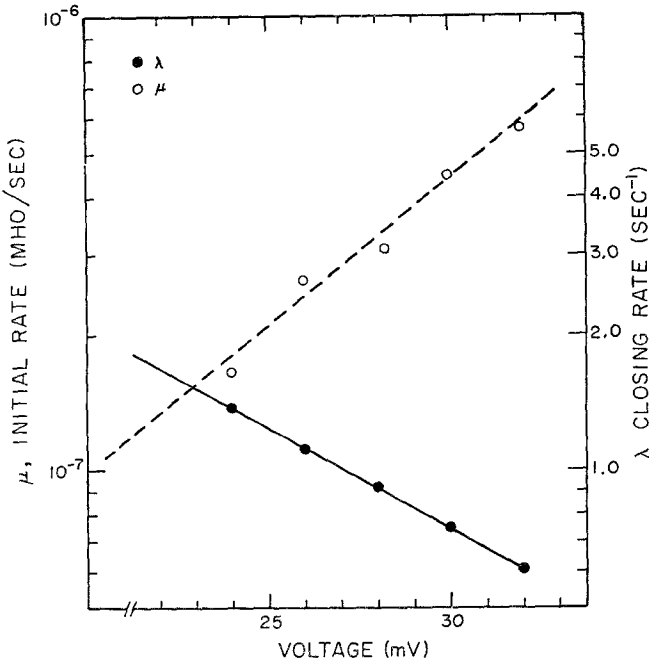

Fig. 17. The initial rate (left-hand scale) and the rate of closing (right-hand scale) for a PE-decane membrane: 1.0 M NaCl 6.5×10^{-7} g/ml alamethicin. Determined from experiments such as that shown in Fig. 16

order of 20 sec^{-1}. Thus, both the forward rate increases and the reverse rate decreases exponentially with increasing voltage, causing a very rapid increase of $\bar{n}$ with increasing voltage:

$$\bar{n} = \frac{\mu}{\lambda} = \frac{\mu_0}{\lambda_0} e^{V\left(\frac{1}{V_\mu} + \frac{1}{V_\lambda}\right)}. \tag{11}$$

Comparison with Eq. (4) shows that

$$\frac{1}{V_\mu} + \frac{1}{V_\lambda} = \frac{1}{V_0}. \tag{12}$$

By varying membrane area in the manner previously mentioned, we have shown that μ is directly proportional to area. This finding implies that μ_0 is a function of the number of entities in the membrane capable of being made electrically conducting by the application of an electric field. It remains to establish that the concept of a pore is applicable at high current levels and that μ and λ actually refer to the rates of formation and disappearance of pores.

Conductance Fluctuations at High Conductance Levels

To find out whether or not the idea of a pore fluctuating between allowed levels is valid in regions where the current is so high that single steps cannot be resolved, we calculate the fluctuation in the conductance expected at high levels. This fluctuation should arise from two sources; the fluctuations in conductance of the individual pores and the fluctuations in the number of pores. Let G be the conductance of the membrane as measured at any given time and G_n the conductance of a membrane constrained to have exactly n pores open.

$$G_n = \sum_{i=0}^{n} \gamma_i \tag{13}$$

where γ_i is the conductance of the i^{th} pore at a particular time. To derive the fluctuations we calculate the average conductance and the average of the square of the conductance. With n constrained, the average of the conductance is

$$\overline{G_n} = \sum_{i=0}^{n} \overline{\gamma_i} = n\bar{\gamma} \tag{14}$$

where $\bar{\gamma}$ is the mean conductance of a pore. If we now let n vary,

$$\bar{G} = \overline{G_n} = \overline{n\gamma}. \tag{15}$$

This result simply says that the average conductance of the membrane is equal to the average number of pores times the average conductance of a pore. We now calculate the average of the square of the conductance. For fixed n we have

$$\begin{aligned} G_n^2 &= \overline{\sum_{i=1}^{n} \gamma_i \sum_{j=1}^{n} \gamma_j} \\ &= n\overline{\gamma^2} + (n^2 - n)\bar{\gamma}^2. \end{aligned} \tag{16}$$

Letting n vary we see that

$$\overline{G^2} = \bar{n}\overline{\gamma^2} + (\overline{n^2} - \bar{n})\bar{\gamma}^2. \tag{17}$$

For a Poisson distribution, $\overline{n^2} - \bar{n}^2 = \bar{n}$; thus, for our distribution

$$\overline{G^2} = \bar{n}\overline{\gamma^2} + (\bar{n}\bar{\gamma})^2. \tag{18}$$

Combining Eqs. (15) and (18) we can now write the variance of the conductance σ_G^2 as

$$\sigma_G^2 = \overline{G^2} - \bar{G}^2 = \bar{n}\overline{\gamma^2}. \tag{19}$$

Table 1. (*a*) 1.0 M NaCl, 6×10^{-7} g/ml alamethicin

Voltage (mV)	$\sigma_G^2/\bar{G}$ (mho)	No. of pores
24	3.25×10^{-9}	57
26	2.16×10^{-9}	109
28	3.48×10^{-9}	164
30	3.71×10^{-9}	264
34	3.38×10^{-9}	664

$\bar{\gamma}=2.5\times10^{-9}$ mho; $\langle\sigma_G^2/\bar{G}\rangle=3.20\pm0.27\times10^{-9}$ mho; $\langle\overline{\gamma^2}/\bar{\gamma}\rangle=3.05\times10^{-9}$ mho.

(*b*) 0.05 M NaCl, 2×10^{-6} g/ml alamethicin

Voltage (mV)	$\sigma_G^2/\bar{G}$ (mho)	No. of pores
67.5	1.00×10^{-10}	10
67.5	3.13×10^{-10}	8.1
70	2.25×10^{-10}	20
70	2.23×10^{-10}	20
72	2.63×10^{-10}	31

$\bar{\gamma}=2.08\times10^{-10}$ mho (extrapolated); $\langle\sigma_G^2/\bar{G}\rangle=2.24\pm0.52\times10^{-10}$ mho; $\langle\overline{\gamma^2}/\bar{\gamma}\rangle=2.55\times10^{-10}$ mho (extrapolated).

Dividing by $\bar{G}$ gives an expression independent of $\bar{n}$:

$$\sigma_G^2/\bar{G}=\overline{\gamma^2}/\bar{\gamma}=\bar{\gamma}+\sigma_\gamma^2/\bar{\gamma}. \tag{20}$$

Note that this expression has, on the right-hand side, only quantities which are properties of a single pore. These quantities are experimentally accessible at low levels. The left-hand side has quantities which can be measured at high levels of current and can be compared with those on the right.

We have measured a number of high level conductance distributions and compared the values obtained for the left side of Eq. (20) with the value of $\overline{\gamma^2}/\bar{\gamma}$ calculated from single pore distributions such as that shown in Fig. 9. The data are summarized in Table 1 for two concentrations of NaCl and show that Eq. (20) is valid. Note that the quantity $\sigma_G^2/\bar{G}$ is not a function of voltage although the number of pores is a function of voltage and has a large experimental range. The effect of the multiplicity of states of a pore is to make the noise ratio $\sigma_G^2/\bar{G}$ larger than it would be if such fluctuations were not allowed.

Eq. (20) shows that the fluctuations arise from two sources: fluctuations in the number of pores and fluctuations in the conductance of individual pores. If there were no fluctuations in the conductances of individual pores, the total fluctuation $\sigma_G^2/\bar{G}$ would have the value $\bar{\gamma}$, the average conductance

of a single pore. Since there are fluctuations within a pore, we have an additional term $\sigma_\gamma^2/\bar{\gamma}$. The value of $\sigma_G^2/\bar{G}$ for 1.0 M NaCl (*see* Table 1) should thus be 0.55×10^{-9} mho greater than the value calculated with fluctuations only in the number of pores. We see in Table 1 for 1 M NaCl that the experimental value is $0.70 \pm 0.27 \times 10^{-9}$ mho greater than the value of the fluctuations calculated by assuming fluctuations only in the number of pores. Thus, Table 1 gives strong evidence that pore formation is the mechanism of voltage dependence, even at higher current levels, and that μ and λ do refer to the rates of creation and destruction of statistically independent pores.

We feel that speculation on the detailed molecular nature of this entity is not warranted at present. Its existence, however, seems well established. In addition, the voltage dependence of the conductance is the same for all measured alamethicin concentrations and salt concentrations. Thus, the same mechanism is almost certainly responsible throughout for the voltage dependence of the conductance. We have identified this mechanism at low levels as pore formation, and have shown in the case of NaCl solutions, that the same mechanism operates at high currents.

Selectivity of the Pores

In the presence of a salt gradient, a current-voltage curve of the sort shown in Fig. 18 can be obtained. The front chamber has 0.005 M KCl and 9×10^{-6} g/ml alamethicin. In the back chamber the concentration of salt is 100 times higher and that of alamethicin seven times lower. The curve shows two characteristic voltages, $V_c^{\text{front}} \simeq +25$ mV, and $V_c^{\text{back}} \simeq -10$ mV. These are the values expected for the salt and alamethicin concentrations on the two sides. Between these two V_c's the conductance is low. Above V_c^{front} it shows a differential negative resistance. However, if we use the usual definition of conductance

$$G = I/(V - V_R) \tag{21}$$

where V_R is the resting voltage (zero current), the conductance as a function of the voltage V rises exponentially as before. This is shown in a semilog plot in Fig. 19. The conductance in the region where V is close to V_c shows discrete levels.

We conclude that near V_R we have a voltage-dependent number of pores and not a voltage dependence of the conductance of a single pore. The value of V_R for a given type of salt thus reflects the selectivity of the pore. The selectivity of the pore may be influenced by the surface charge of the membrane. At pH 5.6 we find the following values of V_R: for NaCl, 13 mV/

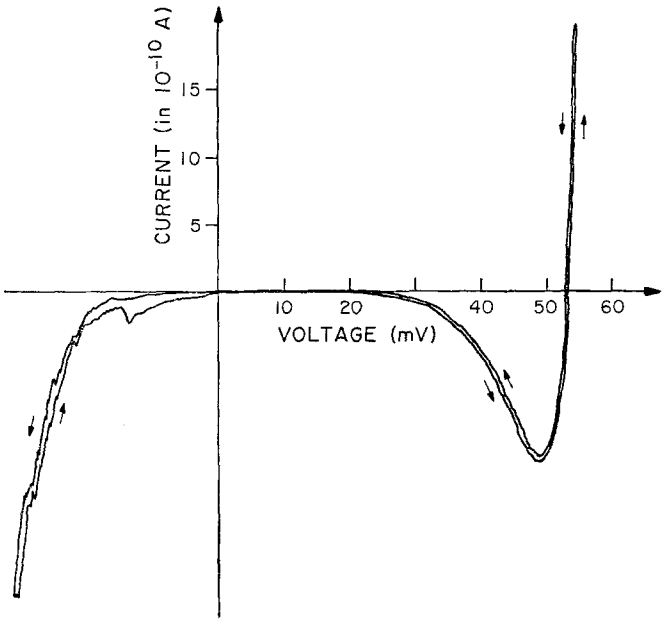

Fig. 18. Voltage-current curve of a PE-decane membrane in a 100:1 KCl gradient. *Back:* 0.005 M KCl and 9×10^{-6} g/ml alamethicin. *Front:* 0.5 M KCl and 6×10^{-7} g/ml alamethicin

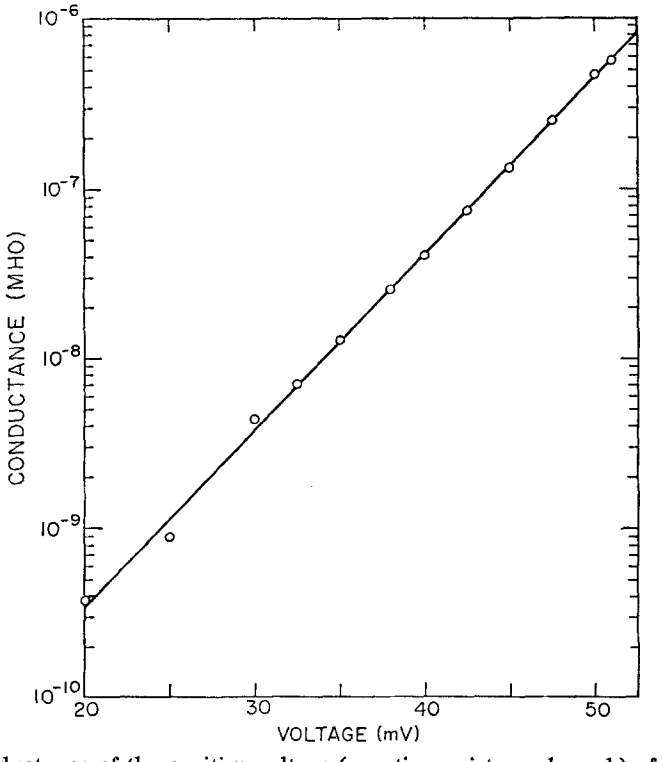

Fig. 19. Conductance of the positive voltage (negative resistance branch) of the curve in Fig. 18. Note that the conductance is an exponential function of voltage with the same form as in Fig. 6 where no differential negative resistance is seen. This finding indicates that the mechanism of voltage dependence is the same in all cases

decade of salt gradient; for KCL, 26.5 mV/decade; and for $CaCl_2$, -26 mV/decade. Positive potentials indicate cation selectivity and negative ones, anion selectivity.

If we assume that the current flow through a pore is given by the usual thermodynamic treatment, and calculate the permeability ratios of the cations relative to chloride, we get $P_{Ca}/P_{Cl} = 0.3$; $P_{Na}/P_{Cl} = 1.6$; $P_K/P_{Cl} = 2.7$. The pore is thus not very ion specific, and it is quite possible that the surface charge of the membrane may be the most important factor determining the selectivity.

Discussion and Summary

Alamethicin does not diffuse readily through PE-decane membranes in which the fatty acid chains are mostly saturated. It adsorbs reversibly to the membrane surface with a time constant of about 2.9 min.

The conductance induced by alamethicin in PE-decane membranes is the sum of the conductances of statistically independent units. These units we have called pores. Even at high conductance levels where there are many pores present, the open pores are, if randomly distributed, widely spaced, about 30 μ apart. It is thus not appropriate to invoke long-range cooperativity to explain the rapid increase of conductance with voltage.

The conductance of an individual pore fluctuates between five levels, ranging from roughly 0.1 to 1.0 nmho in 0.1 M salt. The frequency range of the fluctuations is about 10 to 1,000 Hz, and the levels occur with a characteristic probability distribution, the second and third levels being most likely.

These characteristics of a pore are remarkably independent of voltage, of the alamethicin concentration and of the type of salt. Even the character of the fluctuations is indepedent of these quantities. These results suggest that we are dealing with a sharply defined structure capable of several states of conformation.

The conductances of each level vary with salt concentration and with the viscosity of the bulk solution, and these variations parallel the conductance variations of the bulk solutions. These facts suggest that the pores are rather large. Indeed if we assume a cylindrical shape for the pore and a conductivity of the solution in its interior equal to the conductivity of bulk solution, we can estimate the pore diameter and find it ranges from about 10 to 25 Å for the various levels.

The pores do not show strong selectivity for different ions. We find the permeabilities of Na, K and Ca relative to chloride to be 1.6, 2.7 and 0.3 (from zero current potential measurements). Assuming the concentrations of anion and cation in the pore to be equal, these values give ratios of Na and Ca conductance to K conductance of 0.7 and 0.3, in fair agreement with

0.8 and 0.4, the measured values for the 2nd and 3rd levels. The diffusion time for an ion to move across the pore under the influence of a potential of 100 mV will be about 5×10^{-9} sec. The mean time for an ion to collide with a wall of the pore assuming a diameter of about 10 Å will be 2.5×10^{-10} sec. Thus, an ion can interact with the walls of a pore while moving through, and this interaction could account in part for the small selectivity of the pore for the different ions tested.

The average number of open pores at any one time is governed by the rates of formation and disappearance. Both of these rates depend strongly on the applied voltage. At voltages giving a measurable conductance, the rate of disappearance has a time constant of about 1 sec, a time long compared to the characteristic time of fluctuation. A survey of many scans suggests that pores are formed preferentially in the lowest conductance state (zeroeth level) and also that they disappear preferentially from that state. Thus, a pore, once formed, has a high probability of fluctuating through all its levels before disappearing.

Shortly after the application of a voltage pulse, the conductance will not be given by the number of pores times the average conductance of a pore, but by the conductance of the lowest level times the number of pores. As soon as the pores have had a chance to fluctuate among all their levels, the conductance will approach the product of the number of pores times the average conductance of a pore. All our kinetic measurements were made in this regime. The rate of formation rises e-fold for a 6.7 mV rise in potential on the alamethicin side and the rate of disappearance decreases e-fold for a 9.6 mV rise of potential. The net effect is an e-fold increase in the number of pores at equilibrium for a rise in potential of 3.94 mV.[3]

3 The results of this paragraph explain the observations of Mauro, Nanavati and Heyer (1972). They find an s-shaped kinetic curve of alamethicin-induced current after the application of a voltage pulse. They state that the s-shaped response is over after several milliseconds and it is followed by a response of the form already discussed, namely $g=g_\infty(1-e^{-\lambda t})$. Such an effect is precisely as expected from our results. The time interval of the s-shaped behavior should be on the order of the fluctuation time, a few milliseconds. Our results also quantitatively explain their measurement of a rapid turn-off of the current following a voltage polarity reversal. We estimate that the rate of pore disappearance at zero volts is about 16 sec^{-1} corresponding to a characteristic time of about 60 msec. At a negative voltage of −50 mV, the rate of disappearance would be much faster, about 1,700 sec^{-1}, or a characteristic time of about 500 μsec. Mauro *et al.* (1972) present data on the response of alamethicin-induced conductance to voltage steps. When the voltage is stepped from +50 to −50 mV, the conductance turns off with a characteristic time longer than 200 μsec. This value is quite compatible with our results. They also suggest that alamethicin and salt concentrations affect the relaxation time. This is certainly true and is quantitatively confirmed by our work [*see* in particular, Eqs. (7) and (24)]; however, they have neglected the strong voltage dependence of the rates of formation and disappearance.

The kinetics of formation and disappearance show that any one pore is formed from alamethicin on one side of the membrane. This is so because the rate of formation of a pore from alamethicin on one side is independent of the alamethicin concentration on the other side. It is not clear whether on disappearance of a pore, all of the alamethicin contained in it returns preferentially to the side from which it came.

These findings can be interpreted in terms of a three-state scheme: prepore; activated state; pore. Each of the three states has its own energy level. The energy level of the prepore is the lowest and that of the activated state the highest. The rate of formation will be determined, in part, by the difference in energy between the prepore and the activated state. The number of prepores at a given voltage will be determined by the alamethicin concentration and the salt concentration. The rate of disappearance will be determined in part, by the difference in energy between the pore and the activated state. The equilibrium between pore and prepore will be determined by the difference in energy between the two states. If we assume that the energy of the prepore is not affected by the potential, we can calculate that the energy level of the pore is lowered by kT when the potential is raised by 3.94 mV. From the change in the number of pores with voltage we can write:

$$\exp(-E_{\text{pore}}/kT)=\exp(-E_0/kT+V/3.94)$$

$$E_{\text{pore}}=E_0-\frac{V}{3.94}kT. \tag{22}$$

E_0 is the energy of the pore state relative to the prepore state when no voltage is applied. Similarly, from the voltage dependence of the formation rate, the energy level of the activated state is lowered by 0.58 kT when the potential is raised by 3.94 mV.

These energy relations imply that a positive charge is displaced when a pore is formed. If we assume that the charge is displaced across the entire applied field, we obtain a minimum estimate for the charge. This minimum is six elementary charges. If the same number of charges is moved in the formation of the activated state, they must be moved by a lesser distance, a little more than half that required to reach the pore state.

These energy differences do not depend on salt concentration, suggesting that the charges are not charges filling the bulk of the pore, but are associated with a sharply defined structure. The energy differences are also independent of alamethicin concentration suggesting in turn that the charges may be complexed with a fixed number of alamethicins. Pressman (1968) has shown that alamethicin forms complexes with alkali cations (K, Na, Rb, Cs).

Payne *et al.* (1970) find that alamethicin appears to have only one titratable group with a pK of 5.5. This is most likely the free glutamine carboxyl end which is ionized above pH 5.5. These results tend to confirm our belief that alamethicin acquires a positive charge by complexing with available cations.

The equilibrium concentration of pores increases with the 9th power (9.2 ± 1.1) of the alamethicin concentration and with the 4th (4.2 ± 0.35) power of the salt concentration. These results follow from the dependences of the characteristic voltage on alamethicin and salt concentrations. We know that the number of pores can be written

$$n = n_c e^{\frac{V - V_c}{3.94\,\mathrm{mV}}} \tag{23}$$

where n_c is the number of pores at $V = V_c$ and is independent of voltage. Substituting for V_c from Eq. (3) gives

$$\begin{aligned} n &= n_c \exp\left\{\frac{V}{3.94\,\mathrm{mV}} + \left(\frac{V_{\mathrm{salt}}}{3.94} \ln C_{\mathrm{salt}} + \frac{V_a}{3.94} \ln C_a - \frac{V_i}{3.94}\right)\right\} \\ &= n_c (C_{\mathrm{salt}})^4 (C_a)^9 \exp\left(\frac{V - V_i}{3.94}\right). \end{aligned} \tag{24}$$

V_i is a constant depending on the type of salt and the membrane area.

One may conjecture that this dependence of the number of pores on powers of salt and alamethicin concentrations reflects an equilibrium between salt and alamethicin in solution with a hypothetical structure in the membrane surface, the prepore. The rate of pore formation would then be proportional to the number of prepores present and a voltage-dependent rate factor.

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